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TITLE OF THE INVENTION (280 characters max)

ORGANIC COMPOUNDS

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Respectfully submitted,

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Organic Compounds

The present invention relates to organic compounds, e.g. to a method for modulating regulatory RNA-ligand interactions and assays.

(m)RNA-protein interactions are known to be of central importance in the regulation of eukaryotic gene expression. Tightly controlled processes such as pre-mRNA splicing, nuclear export, message degradation and translatability are essentially dependent on the recognition of cis acting (m)RNA elements by protein mediators. Beside generic interactions like cap- or poly(A) binding, this often occurs in a highly specific and controlled manner. Several examples have been described wherein recognition relies not only on pure primary structure but also involves higher order sequence properties, e.g. (m)RNA secondary structure. Many disease relevant genes are regulated predominantly on post transcriptional level. Hence, targeting regulatory RNA-ligand interactions, e.g. mRNA-protein interactions, through (m)RNA secondary structure manipulation may serve as a novel strategy for therapeutic intervention.

While long-lived (m)RNAs are buffered against rapid changes with respect to the production of a specific gene product, a short (m)RNA half-life is essential to permit timely adjustments to changing physiological conditions and to cellular, often receptor-mediated, signals. It has been found that half-lives of messages from inflammatory cytokines, growth factors and several proto-oncogenes are subjected to tight control mechanisms often mediated by AU-rich elements (= AREs).

(m)RNAs from many disease-relevant early-response genes (ERGs) are targeted for specific degradation by the presence of AREs in the 3'-untranslated region (UTR). Because of the relevance of proteins encoded by ARE-containing mRNAs, this element must be considered a pivotal target for anti-inflammatory therapies but also for targets of essentially different relevance like proto-oncogenes such as e.g. c-fos, c-myc as well as bcl-2. Particularly the mRNAs of cytokines and proto-oncogenes are targeted in cis for degradation by AREs in their 3`-UTR, mediated by trans-acting factors or proteins binding to them.

AREs are basically characterized by the presence of the pentameric consensus motif AUUUA. However, the ARE-sequences differ from each other by the arrangement and number of these pentanucleotides. Moreover, the number, length and position of the ARE within the 3'-UTR is highly variable. Whereas multiple AUUUA sequences in close proximity or AU-rich regions have been implicated in mRNA instability, isolated AUUUA sequences

may in contrast have other regulatory functions, for example in translation and mRNA localization. ARE-directed (m)RNA decay (degradation) is initiated by rapid removal of the poly-(A)tail, followed by degradation of the message corpus (see e.g. Chen C.Y et al., Trends Biochem. Sci. 1995, 20(11):465-70).

To date, several cytoplasmic (m)RNA-binding proteins have been identified to specifically interact with the ARE, whereas their binding shows either stabilizing, destabilizing or shuttling effects. Among these, the human ELAV (embryonic-lethal/abnormal-vision)-protein HuR (Hu-Antigen R) is proposed to be the central (m)RNA stabilizing protein involved in ARE-mediated mRNA degradation pathways (see e.g. Peng S.S. et al., EMBO J. 1998, 17(12):3461-70).

HuR is a 36kD protein of the RRM (RNA recognition motif)-superfamily which, in addition to stabilize short-lived (m)RNA by its ARE-binding activity in the 3'-UTR, has been shown to redistribute between the nucleus and the cytoplasm. Therefore, it is supposed to bind its cognate mRNAs in the nucleus and then escort them through the nuclear pore. It provides protection from degradation during and after export to the cytoplasm, thereby resulting in immediate up-regulation of the corresponding gene. The large family of AU-rich containing mRNAs associated with HuR-mediated regulation includes e.g. IL-3, c-fos, c-myc, GM-CSF (granulocyte/monocyte-colony stimulating factor), AT-R1 (angiotensin-receptor 1), Cox-2 (cyclooxygenase-2), IL-8 or TNF-α (see e.g. Hel Z. et al., Nucleic Acids Res. 1998, 26 (11): 2803-12).

The processes mediating up- and down regulation of immune mediators like IL-2, Cox-2 or TNF-α are key mechanisms in the immune response and represent an important target for immune intervention and anti-inflammatory therapies.

Despite the fact that a broad class of (m)RNAs uses such generic type of regulatory element, previous studies have provided evidence that the ARE of a particular mRNA subsides a remarkably specific response to cell signaling. Hence, it appears to be feasible to identify (m)RNA-specific functional inhibitors by targeting individual AREs. This suggests that a great variety of disease relevant mediators, including proto-oncogenes, inflammatory cytokines and viral proteins, can be assayed based on the common regulatory principle of ARE- and HuR-mediated (m)RNA-stabilization and nuclear export.

One of the key questions is how a dozens of proteins with essentially only one positive regulator can achieve such a specific regulation of up to 3000 genes. Even more, it is not yet known whether the binding of HuR to its target ARE is in vivo indeed the specificity determining

step. Understanding how specificity is accomplished at the molecular level may be a prerequisite for an exploitation of (m)RNA stability pathways in drug discovery.

The complex formation of a (m)RNA, e.g. an ARE-containing (m)RNA, with a ligand, e.g. a protein, e.g. an HuR protein, in consequence induces the expression of various disease causing/mediating substances, e.g. inflammatory acting substances, such as cytokines, growth factors, proto-oncogenes or viral proteins. Agents which inhibit such a complex formation may thus prevent the expression of such substances, e.g. such agent may prevent (inhibit) or reduce the expression of inflammation mediating substance. Therefore such agents (inhibitors) may be used in the treatment of various diseases, e.g. diseases mediated by cytokines, growth factors, proto-oncogenes or viral proteins.
The importance of (m)RNA secondary structure for (m)RNA-ligand interactions can be demonstrated directly. However, this requires the concomitant detection of both (m)RNA secondary structure and ligand binding affinity, which may be intricate and which may require a complex experimental setup.

(m)RNA secondary structure is known to be of central importance in the regulation of many cellular processes such as pre-mRNA processing, metabolic processes, or (m)RNA-protein interactions vital for viral lifecycles. In particular, we have recently shown that it is the local conformation of the AU-rich element (ARE) which controls (m)RNA stability via interaction with the RNA-binding protein HuR. This tightly controlled process determines the expression of approximately 3,000 genes (see e.g. Bakheet T. et al., 2001, Nucl.Acid.Res.29 (1): 246-54 and Bakheet T. et al., 2003, Nucleic Acid Res. 31(1): 421-3) including a number of disease relevant proteins and pharmaceutical targets in cancer, inflammatory, viral, allergic, vascular and infectious diseases. Hence, targeting such regulatory (m)RNA-protein interactions through (m)RNA secondary structure manipulation could serve as a novel strategy for therapeutic intervention.

In one aspect the present invention provides a method for modulating regulatory RNA-ligand interactions comprising

(a) defining and selecting a secondary structure element of an RNA molecule which is required for the recognition by a ligand, e.g. protein,

(b) calculating the thermodynamic probability of the secondary structure element of step a) in the secondary structure ensemble of said RNA,

(c) calculating the thermodynamic probability of the secondary structure element of step a) in the secondary structure ensemble of said RNA hybridized to a reverse complementary oligonucleotide,

(d) determining an oligonucleotide that changes the thermodynamic probability of said secondary structure element beyond a defined probability threshold,

(e) providing an oligonucleotide as determined in step (d),

(f) hybridizing an RNA comprising said secondary structure element of step (a) to an oligonucleotide of step (e), and

(g) determining the effect of said hybridization on the thermodynamic probability of said secondary structure element.

RNA as used herein includes all kind of RNAs, e.g. ribosomal RNA (rRNA), transfer RNA (tRNA) or messenger RNA (mRNA), e.g. of a cytokine, growth factor, interleukin, cyclin, apoptotic protein, hormone, differentiation factor or a viral protein, preferably a mRNA, e.g. including ERGs. For example, the mRNA is an ARE-containing mRNA.

In a preferred aspect of the present invention the RNA is a mRNA, e.g. TNF-α mRNA or mRNA of interleukin 2 (IL-2).

An oligonucleotide (e.g. an opener) of the present invention comprises a sequence which is reverse complementary to a target RNA, e.g. a mRNA. The oligonucleotide may be an oligodesoxyribonucleotide or an oligoribonucleotide. The length of the oligonucleotide depends on the target RNA and on the desired sequence specificity, preferably the oligonucleotide has a length of 10 to 200 nucleotides, such as 10 to 100, preferably 10 to 50, such as about 20 nucleotides.

Hybridization of an oligonucleotide of the present invention to the target RNA changes the thermodynamic probability of a secondary structure element beyond a defined threshold, e.g. a threshold defined as a value which is at least 2 times higher than the value of unhybridized RNA or a value which is at least 0.5 times lower than the value of the unhybridized RNA.

The above described method is hereinafter defined as "method of the present invention". An oligonucleotide as described above is hereinafter defined as "an oligonucleotide of the present invention".

A ligand is a molecule which binds to a regulatory RNA molecule. It may be an RNA, DNA or a protein, e.g. a protein, such as an ARE binding protein, e.g. of the ELAV family, such as ELAVL1 (HuR), HuB, HuC, HuD, e.g. ELAVL1.

A secondary structure element of an RNA is a sequence pattern comprised in the RNA with a defined secondary structure. Such a secondary structure element is involved in the interaction with a ligand. A change in the conformation may have an impact on the interaction of said element with a ligand, e.g. the interaction of a ligand with the RNA may be enhanced (boosted) or lowered (silenced).

In another aspect the present invention provides a method for defining and selecting a secondary structure element which method comprises

(a) providing an RNA-binding ligand LIG,

(b) providing at least 3 different RNAs of sequences s_{M1} s_{M2}, and s_{M3}, each of s_{M1} s_{M2}, and s_{M3} having the same sequence binding motif MOT for the ligand LIG, but each differing in the secondary structure,

(c) calculating each $p(s_M,\sigma_i)$ for each s_{M1} s_{M2}, and s_{M3} which is the probability that a structure contains at least one MOT in a particular test conformation σ_i, e.g. calculating each $p(S_M,\sigma_i)$ according to the equation [2], as set out below,

(d) measuring each macroscopic affinity constant Kd_{app} for the binding of LIG to each of s_{M1} s_{M2}, and s_{M3},

(e) determining for any test conformation σ_i whether the correlation between each of Kd_{app} and $p(s_M, \sigma_i)$ for each of s_{M1} s_{M2} and s_{M3} follows the dependency

$Kd_{app} \sim 1 / p(s_M, \sigma_i)$,

and

(f) choosing a secondary structure element of conformation σ_a which follows said dependency.

If the dependency as set out in step (e) is fulfilled, the secondary structure element of said RNA molecule is of importance in the binding process with a ligand LIG, e.g. a protein.
The method according to the present invention is thus appropriate to find out

- whether a secondary structure element is of importance in the binding process of an RNA to a ligand LIG, e.g. a protein; and

- which secondary structure elements are of importance in the binding process.

An RNA-binding ligand LIG, e.g. a protein, is understood to include any ligand, e.g. protein, which binds via a binding motif MOT to said RNA, e.g. mRNA, e.g. including an ELAVL1 (=HuR)-protein, wherein the binding is dependent on a secondary structure element of said mRNA.

In another aspect of the present invention the method for defining and selecting a secondary structure element further comprises the steps of

(g) determining the fundamental affinity constant Kd_{fund} of LIG to MOT in an active conformation σ_a based on the equation $Kd_{app} = Kd_{fund} / p(s_M, \sigma_i)$, and

(h) (optionally) providing, e.g. novel, sequences with a defined Kd_{app} for the ligand LIG.

As soon as K_{fund} is determined by a method as described above, K_{fund} may be used as a basis to design novel sequences with a pre-defined Kd_{app} for the ligand LIG, e.g. a protein, i.e. the secondary structure element of the RNA as confirmed to be of binding-relevance to LIG according to the present invention may be inserted in other RNA-structures as a binding motif MOT. This may be done by pure calculation without further measurements.

In another aspect the present invention provides a method of the present invention comprising the steps (a) to (e) and further comprising step

(i) screening for additional mRNAs bound by the ligand LIG based on the occurence of MOT in an active conformation σ_a.

RNA molecules, e.g. mRNA, of equal primary sequence S_M may fold into many different secondary structures S_{M1}, S_{M2}, S_{M3}, etc., the frequency of each structure is dependent on its stability, i.e. its energy. If we anticipate that a ligand, e.g. a protein binds only to RNA molecules, e.g. mRNA, which adopt a specific secondary structure element and assume a 1:1 binding mechanism, the interaction can be described as Kd_{fund} wherein Kd_{fund} is expressed in the following equation

$$Kd_{fund} = [RNA_{acc}] \cdot [protein_{free}] / [complex] \qquad (I)$$

In equation I, [protein], [complex] denote the respective equilibrium concentrations, and

$[RNA_{acc}]$ is the concentration of free RNA molecules adopting the fold required for binding; that means RNA molecules having a secondary structure element required for binding to a ligand LIG, e.g. a protein.

If the secondary structure equilibrium is reached sufficiently fast, the concentration $[RNA_{acc}]$ is expressed in the following equation

$$[RNA_{acc}] = [RNA_{free}] \cdot p(s_M, \sigma_i) \qquad (II)$$

In equation (II), $[RNA_{free}]$ is the concentration of unbound RNA in the equilibrium and $p(s_M, \sigma_i)$ is the thermodynamic probability of structures containing at least one sequence binding motif MOT in a particular test conformation σ_i.

The algorithm to calculate the probability $p(s_M, \sigma_i)$ is derived from the Vienna RNA package, see e.g. Hofacker I.L. et al., Monatsh. Chemie 125, 167, 1994, and the examples of the present application.

An experimental determination of the binding affinity between mRNA and protein does not take into account a possible discrepancy between $[RNA_{acc}]$ and $[RNA_{free}]$ and will consequently overestimate the dissociation constant (underestimate the affinity) Kd_{app}, which Kd_{app} may be expressed by the following equation:

$$Kd_{app} = [RNA_{free}] \cdot protein_{free}] / [complex] \qquad (III)$$

wherein $[RNA_{free}]$, $[protein_{free}]$ and [complex] are as defined above.

If $[RNA_{free}]$ is substituted according to equation II by $[RNA_{acc}] / p(s_M, \sigma_i)$ and inserted in equation I, then Kd_{app} can be expressed by the following equation:

$$Kd_{app} = Kd_{fund} / p(s_M, \sigma_i) \qquad (IV)$$

We have confirmed that, if a specific mRNA secondary structure element is required for the binding to the ligand, e.g. a protein, a correlation between measured macroscopic affinity data and computed thermodynamic probabilities of a putatively required secondary structure element will follow the deduced reciprocal relationship expressed in equation (IV).

According to the present invention

- a sequence binding motif MOT is a known primary sequence which is part of the RNA sequence S_M and which is required for the binding to the ligand LIG, e.g. a protein,
- a test conformation σ_a is defined as a set of base pairs (positions i, j) where both or either of i, j are within the sequence binding motif MOT of the RNA sequence.

Such base pairs must fulfill the non-pseudoknot condition. This implies that, if the bases are numbered from 5' to 3', any pair of base pairs ((i, j), (k, l)), (i<k), are either successive (i<j<k<j) or nested (i<k<l<j) but not cross (i<k<j<l).

Macroscopic affinity constants Kd_{app} for the binding of a ligand LIG, e.g. a protein, to a given RNA, e.g. mRNA, may be determined according to known methods, e.g. methods as conventional, such as e.g. EMSA (= electrophoretic mobility shift assay), fluorescence spectroscopy with a particular focus on applications with single molecule sensitivity e.g. Fluorescence Correlation Spectroscopy (FCS), Fluorescence Intensity Distribution Analysis (FIDA), or applications based on the determination of Fluorescence Anisotropy or Fluorescence Resonance Energy Transfer (FRET), e.g. as described in Kask P. et al, Biophys. J. (2000) 78 (4), 1703-1713.

In addition to a qualitative determination of the influence of the RNA secondary structure on the interaction with a ligand, e.g. a protein, the method of the present invention allows also quantitative applications. Kd_{fund}, which is often not experimentally ascertainable, can be determined by curve fitting of good quality apparent macroscopic binding data (Kd_{app}) to RNA structures of variable affinity

On the other hand, the calculation of $p(s_M, \sigma_a)$ for novel sequences combined with the pre-existent experimental assessment of Kd_{fund} allows to predict affinities to certain RNA sequences. Moreover, this approach permits the design of artificial sequences with a specific affinity. This is an important prerequisite for the dissection of the mRNA recognition mechanism by a protein, e.g. HuR.

The potential to predict affinities to a certain regulator and to dissect how the affinity can be manipulated will be of prime significance for target selection within a target platform. The knowledge that a particular secondary structure element is responsible for recognition by a regulatory protein is e.g. of immediate relevance for assay development.

In another aspect the effect of hybridization, which is determined in a method of the present invention, is selected from the group consisting of secondary RNA structure, tertiary RNA structure, RNA-ligand affinity, RNA oligo- or multimerization, ligand oligo- or multimerization, conformational change of the ligand, efficiency of a downstream effect of RNA-ligand recognition, RNA splicing, covalent RNA modifications, RNA localization, RNA stability, RNA translation and protein expression profiles.

In another aspect the RNA is an IL-2 mRNA, the ligand is ELAVL1-protein and the oligonucleotide has a sequence selected from the group consisting of
SEQ ID No 1: AAGGCCTGATATGTTTTAAG,

SEQ ID No 2: AATATAAAATTTAAATATTT,
SEQ ID No 3: TAGAGCCCCTAGGGCTTACA,
SEQ ID No 4: TGAAACCATTTTAGAGCCCC,
SEQ ID No 5: AAGGCCUGAUAUGUUUUAAG,
SEQ ID No 6: AAUAUAAAAUUUAAAUAUUU,
SEQ ID No 7: UAGAGCCCCUAGGGCUUACA,
and
SEQ ID No 8: UGAAACCAUUUUAGAGCCCC.

In another aspect the RNA is a TNF-α mRNA, the ligand is ELAVL1-protein and the oligonucleotide has a sequence selected from the group consisting of

SEQ ID No 9: TCGGCCAGCTCCACGTCCCG,
SEQ ID No 10: TCTGGTAGGAGACGGCGATG;
SEQ ID No 11: ACGGCGATGCGGCTGATGGT;
SEQ ID No 12: TTCTGGAGGCCCCAGTTTGA,
SEQ ID No 13: ATTCCAGATGTCAGGGATCA, and
SEQ ID No 14: ATCACAAGTGCAAACATAAA.

In another aspect the present invention provides the use of a method of the present invention for manipulating the expression of a gene by altering the secondary structure of the corresponding RNA, e.g. up- or downregulating the expression of a gene.

A perfect example for the controlled manipulation of the expression of a gene is e.g. one particular ARE mRNA out of the approximately 3,000 ARE controlled (early response) genes. We have recently described that binding of the positive regulator HuR to an ARE mRNA is determined by the presence of its binding site NNUUNNUUU in single stranded conformation. The method of the present invention allows to provide oligonucleotides which act as modulators in that they maximize or minimize the accessibility of the HuR binding site NNUUNNUUU within a particular target mRNA. By specifically increasing or reducing the complex formation of ELAVL1 (=HuR) with the targeted mRNA, the expression of the corresponding gene may be boosted, alleviated or silenced (up- or down regulated). Hence, the exemplaric oligonucleotides that change the thermodynamic probability of a secondary structure element of an RNA which is required for the recognition by a ligand, e.g. protein (also called mRNA modulators) as specified herein can immediately be used as tools for

mechanistic studies on HuR regulated mRNAs and specific assay design: Assuming that the cell uses modulators like small RNAs or proteins to induce mRNA structure changes strongly related or similar to the oligonucleotides as provided by the present invention, their application to design target specific mRNA stability assays is possible. The oligonucleotides of the present invention lead to a structural rearrangement, which can be detected e.g. by standard spectroscopic methods. Small molecules or substances can be tested for either inhibiting or enhancing this effect by binding to a specific conformation in the mRNA structure during rearrangement.

In a further aspect the present invention provides an assay for identifying an agent that modulates the effect of the hybridization of an RNA molecule to an oligonucleotide comprising

(a) hybridizing an RNA comprising a secondary structure element which is required for recognition by a ligand to an oligonucleotide that changes the thermodynamic probability of said secondary structure element beyond a defined probability threshold in the presence and in the absence of a candidate compound,

(b) determining the effect of hybridization of said RNA to said oligonucleotide in the presence and in the absence of said candidate compound,

(c) identifying an agent which modulates the effect of hybridization.

In another aspect the present invention provides an assay for identifying an agent that mimics the effect of hybridization of an RNA molecule to an oligonucleotide comprising

(a) hybridizing an RNA comprising a secondary structure element which is required for recognition by a ligand to an oligonucleotide that changes the thermodynamic probability of said secondary structure element beyond a defined probability threshold

(b) hybridizing an RNA comprising a secondary structure element which is required for recognition by a ligand to a candidate compound which is expected to have a similar effect as the oligonucleotide,

(c) determining the effect of hybridization for steps (a) and (b), and

(d) identifying an agent which mimics the effect of hybridization of step (a).

Assay(s) as described above are hereinafter referred to "assay(s) of the present invention".

In a further aspect the effect of hybridization is determined by measuring a signal which is related to the effect of hybridization as described above.

In a preferred aspect the RNA in the assay(s) of the present invention is a mRNA.

In a further aspect the RNA, the ligand and the oligonucleotide in the assay(s) of the present invention are as described above.

In another aspect the present invention provides the use of an assay of the present invention for high throughput screening.

In another aspect the present invention provides an agent identified by an assay of the present invention for use as a pharmaceutical.

In a further aspect the present invention provides an oligonucleotide that changes the thermodynamic probability of a secondary structure element beyond a defined probability threshold identified by a method of the present invention.

In another aspect the present invention provides an oligonucleotide having a sequence selected from the group consisting of

SEQ ID No 9: TCGGCCAGCTCCACGTCCCG,
SEQ ID No 10: TCTGGTAGGAGACGGCGATG;
SEQ ID No 11: ACGGCGATGCGGCTGATGGT;
SEQ ID No 12: TTCTGGAGGCCCCAGTTTGA,
SEQ ID No 13: ATTCCAGATGTCAGGGATCA, and
SEQ ID No 14: ATCACAAGTGCAAACATAAA.

In another aspect the present invention provides a pharmaceutical composition comprising an agent identified by assay(s) of the present invention or an oligonucleotide of the present invention beside at least one pharmaceutical excipient, e.g. appropriate carrier and/or diluent, e.g. including fillers, binders, disintegrators, flow conditioners, lubricants, sugars and sweeteners, fragrances, preservatives, stabilizers, wetting agents and/or emulsifiers, solubilizers, salts for regulating osmotic pressure and/or buffers.

In another aspect the present invention provides a pharmaceutical composition of the present invention, further comprising another pharmaceutically active agent.

Such compositions may be manufactured according, e.g. analogously to a method as conventional, e.g. by mixing, granulating, coating, dissolving or lyophilizing processes. Unit dosage forms may contain, for example, from about 0.5 mg to about 1000 mg, such as 1 mg to about 500 mg.

For use as a pharmaceutical, an agent or an oligonucleotide of the present invention includes one or more agents or oligonucleotides, e.g. a combination of agents or oligonucleotides.

The pharmaceutical compositions of the present invention may be used for the treatment of a disorder having an etiology associated with the production of a substance, e.g. an inflammatory acting (causing/enhancing) substance, selected from the group consisting of cytokine, growth factor, proto-oncogene or viral protein.
Preferably said substance is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-8, GM-CSF, TNF-α, VEGF, AT-R1, Cox-2, c-fos and c-myc.
Treatment includes treatment and prophylaxis.
For such treatment, the appropriate dosage will, of course, vary depending upon, for example, the chemical nature and the pharmakokinetic data of an agent or an oligonucleotide of the present invention employed, the individual host, the mode of administration and the nature and severity of the conditions being treated. However, in general, for satisfactory results in larger mammals, for example humans, an indicated daily dosage is in the range from about 0.01 g to about 1.0 g, of an agent or an oligonucleotide of the present invention; conveniently administered, for example, in divided doses up to four times a day.
An agent or an oligonucleotide of the present invention may be administered by any conventional route, for example enterally, e.g. including nasal, buccal, rectal, oral, administration; parenterally, e.g. including intravenous, intramuscular, subcutanous administration; or topically; e.g. including epicutaneous, intranasal, intratracheal administration;
e.g. in form of coated or uncoated tablets, capsules, injectable solutions or suspensions, e.g. in the form of ampoules, vials, in the form of creams, gels, pastes, inhaler powder, foams,

tinctures, lip sticks, drops, sprays, or in the form of suppositories.
An agent or an oligonucleotide of the present invention may be administered in the form of a pharmaceutically acceptable salt, e.g. an acid addition salt or metal salt; or in free form; optionally in the form of a solvate. An agent or an oligonucleotide of the present invention in the form of a salt exhibit the same order of activity as the compounds of the present invention in free form; optionally in the form of a solvate.

An agent or an oligonucleotide of the present invention may be used for pharmaceutical treatment according to the present invention alone or in combination with one or more other pharmaceutically active agents.
Combinations include fixed combinations, in which two or more agents or oligonucleotides of the present invention are in the same formulation; kits, in which two or more agents or oligonucleotides of the present invention in separate formulations are sold in the same package, e.g. with instruction for co-administration; and free combinations in which the agents or oligonucleotides of the present invention are packaged separately, but instruction for simultaneous or sequential administration are given.

In another aspect the present invention provides the use of an oligonucleotide of the present invention for manipulating regulatory RNA-ligand interactions.

In another aspect the present invention provides the use of an oligonucleotide of the present invention for influencing the stability of an RNA molecule, e.g. of a mRNA, e.g. stabilizing an ARE containing mRNA.

Description of the figures

Figure 1:

Schematic illustration of the opener effect

Without the oligonucleotide, which acts as an "*opener*", HuR binding is impaired by inaccessibility of the binding site. Upon hybridization of the oligonucleotide to the target mRNA (mRNA α), selective modulation of the local secondary structure within the α mRNA leads to a presentation of the HuR binding site in accessible conformation, resulting in mRNA α stabilization, without affecting any other HuR target mRNA (e.g. mRNA ß)

Figure 2:

Design of IL-2 opener oligonucleotides

HuR binding site accessibility *p(ssNNUUNNUUU)* is shown in dependence of IL-2 3'UTR hybridization to a reverse complementary 20mer oligonucleotide at a given position. The IL-2 ARE is indicated as a white box, NNUUNNUUU HuR binding sites are shown in bold. A significant increase in the accessibility is restricted to discrete "hotspots" in proximity of the HuR binding sites, characterizing potential *openers*. The location of experimentally tested oligonucleotides (*opener* molecules) O_1 and O_2 is indicated as well as those of negative controls (N_1, N_2) (sequences are specified in Table 1). O_1 targets primarily the HuR binding site within the ARE, O_2 is directed to the second NNUUNNUUU motif. Due to the assumption of saturated hybridization to the oligonucleotides (openers), predicted Kd_{app} values based on *p(ssNNUUNNUUU|opener)* are lower limits of experimentally observable values.

Figure 3

Minimum free energy (MFE) secondary structure of the IL-2 3`UTR and the IL-2 3`UTR hybridized to opener O

As exemplified by the MFE conformation of the complex, the oligonucleotide acting as an opener shifts the equilibrium towards conformations with accessible (i.e.single stranded) NNUUNNUUU elements – following the model sketched in Figure 1. This results in an increase in the apparent HuR binding affinity in presence of the oligonucleotide acting as an opener (IL-2 3`UTR concentration = 2.5 nM, O1 concentration = 25 nM). MFE secondary structures for 3`UTR – oligonucleotide complexes are computed using the program Cofold.

Figure 4

IL-2 mRNA openers increase endogenous HuR IL-2 mRNA association

Human PBMC are activated with anti-CD3 or anti-CD3 plus anti-CD28 antibody. HuR-bound IL-2 mRNA is co-immunoprecipitated from lysates after or without treatment with oligonucleotides specified as *openers* O_1 or O_2 and quantified by real time RT-PCR. IL-2 mRNA amounts are normalized to the levels in anti-CD3 activated but untreated cells (open bars). Oligonucleotides are added to 2.5 µM (solid bars) or 10 µM concentration (hedged bars). Upon stimulation with CD28, IL-2 mRNA HuR association is increased 2.4 fold. The same level can be reached without CD28 stimulation by the action of O_1 and O_2 at 2.5 µM concentration. This level can be even exceeded by further increasing the oligonucleotide concentration.

Figure 5

Designed IL-2 mRNA openers inhibit IL-2 mRNA degradation

Degradation of endogenous IL-2 mRNA is monitored in human PBMC lysates. Upon addition of Mg^{2+} (t = 0 min), the amount of remaining IL-2 mRNA is quantified over time in the

presence and in the absence of O_1, O_2 or N_2 by quantitative real-time RT-PCR (all data normalized to the levels at timepoint t = 0 min). Data are fitted to a single exponential decay (solid line: no *opener*; dashed line: N_2). IL-2 mRNA is rapidly degraded at a halflife of $t_{1/2}$ = 8.34 (± 0.96) min (A) or $t_{1/2}$ = 8.44 (± 1.98) min (B) without any *opener* (open circles), as well as in presence of 10 µM of negative control N_2 (panel A, crosses, $t_{1/2}$ = 6.82 (± 1.96) minutes). Addition of *opener* O_1 at 10 µM concentration (A) leads to an arrest of the degradation over a period of 15 minutes (full circles), which gradually reverts into decay at prolonged incubation. At 40 µM concentration (B), O_1 blocks the degradation over the entire incubation time of 70 minutes (panel B). Although it targets another HuR binding site, O_2 shows a similar stabilizing effect (panel B, open stars; O_2 at 40 µM concentration).

Figure 6

Correlation between accessibility of NNUUNNUUU within AREs and observed Kd_{app}.

Experimentally determined affinities of HuR to the AREs of Cox-2 (1), IL-1β (2), IL-2 (3), IL-4 (4), IL-8 (5), $(AUUU)_3A$ (10), $(AUUU)_4A$ (11), $(AUUU)_5A$ (12), $(CUUU)_4C$ (13) and to the ARE of TNF-α (6) as well as strategically designed variants TNF-α-WT (7), TNF-α-NZW (8) and TNF-α-mut (9) are plotted against the corresponding accessibilities of the HuR sequence binding motif, p (ssNNUUNNUUU).

In the following examples all temperatures are given in degree Celsius (°) and are uncorrected.

The following ABBREVIATIONS are used:

PBMC	peripheral blood mononuclear cells
IPTG	Isopropyl-ß-D-thiogalaktopyranoside
MFE	minimum free energy
ORN	oligoribonucleotide
RT	room temperature
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TFA	trifluoracetic acid
THF	tetrahydrofuran
TEAAc	triethylammonium acetate
TMR	carboxytetramethylrhodamine

EXAMPLES

EXAMPLE 1

a) Preparation of fluorescently labeled RNA fragments:

RNA synthesis is performed on an Applied Biosystems 394A synthesizer using either 5'-O-dimethoxytrityl-2'O-triisopropyloxymethyl (TOM)- protected β'-cyanoethyl-(N,N-diisopropyl-) nucleotide phosphoramidites (0.1 M solutions in anhydrous acetonitrile; Glen Research) and the appropriate 2'TOM-protected nucleotides immobilised on CPG (controlled pore glass), adopting published procedures (see e.g. Chaix C. et al., Nucleic Acids Symp. Ser. 45, 1989 or Scaringe S.A. et al., Nucleic Acids Res. 18, 5433, 1990) and manufacturer's protocols. In the final step of each synthesis, an aminolinker, (6-(4-monomethoxytritylamino)hexyl-(2-cyanoethyl)-(N,N-diisopropyl)), is attached to the 5'-OH group of the ORN in order to allow the coupling with a range of different dyes in a post-synthesis reaction.

The synthesised ORNs are cleaved from the support, base-and phosphate-deprotected with ammonia-saturated ethanol for 17 hours at 40°. The 2' silyl-protection groups are removed by reaction in 1.1 M tetrabutylammonium fluoride/THF for 15 hours at RT. The deprotected ORNs are purified by denaturing polyacrylamide gel electrophoresis following standard protocols.

For the determination of the concentration, the exact molar extinction coefficient at 260 nm is determined according to e.g. Gray D.M. et al., Methods in Enzymology 246, 19, 1995 and concentrations are calculated based on the measured UV-absorption at 260 nm, according to Beer's Law. The purity and quality of the fragments is controlled by analytical RP-HPLC on a VYDAC C_{18} column with elution in a gradient of 0 to 100 % B in 45 minutes (A: 5% 0.1 M TEAAc pH 7.0, 95 % CH_3CN; B: 50 % 0.1 M TEAAc pH 7.0, 50 % CH_3CN) and UV-detection at 260 nm.

TMR as one of the best characterised and photostable dyes is attached to the aminogroup introduced with the 5-'terminal linker by a standard reaction of the primary amine with the succinimidylester-activated fluorophore leading to the formation of a stable carboxamide. Briefly, the RNA is reacted with a 20-30-fold molar surplus of TMR-NHS (5-carboxy-tetramethylrhodamine-N-hydroxy-succinimidylester; Amersham Pharmacia Biotech) in 100 – 250 mM $Na_2CO_3/NaHCO_3$, pH 8.3 for at least 2 hours at RT and protected from light. Unreacted dye is hydrolysed by addition of 1.5 M hydroxylamine-hydrochloride to a 50-fold molar excess and incubation for further 30 minutes. After labeling, the RNA is separated from the free dye by gel filtration and further purified by preparative RP-HPLC as described above. Peaks containing labeled RNA are collected and any remaining TEAAc is removed by

gel filtration. The concentration of the labeled RNA is determined by UV absorption spectroscopy as described above but with correction for the dye absorption at 260 nm.

b) Preparation of recombinant human HuR:

Recombinant full-length human HuR and a shortened variant encompassing the first two RRMs (RNA Recognition Motifs) only (HuR12) are prepared using the IMPACT™ -CN system (New England Biolabs) as follows: the nucleotide sequence encompassing amino acids 1-326 (full-length HuR) or 1 – 189 (HuR12) of the CDS [Celera identifier CRA hCP40023.2] is PCR-amplified from cDNA prepared from activated human T-Lymphocytes and cloned directionally into the NdeI and SapI sites of the vector pTXB1, allowing C-terminal fusion with an intein- CBD (Chitin binding fomain) tag without insertion of any additional amino acid.

These constructs are transformed into the host strain E.coli ER2566 (New England Biolabs) for protein expression. The DNA sequence of the constructs recovered from positive clones is verified by automated DNA sequencing on an ABI 310 instrument, according to the manufacturer's protocol.

Expression of the fusion proteins is induced by addition of 1 mM IPTG to a bacterial culture grown to late-logarithmic phase in LB (Lurea Bertani) broth and allowed to proceed for 6 hours at 28 °. The bacterial cells are lysed by successive freezing/thawing cycles in a buffer of 20 mM Tris/Cl (Tris(hydroxymethyl)aminomethane) pH 8.0, 800 mM NaCl, 1 mM EDTA (N,N,N',N'-Ethylenediaminetetraacetic) and 0.2 % Pluronic F-127 (Molecular Probes). After DNA digestion, the bacterial lysates are cleared by ultracentrifugation and the fusion protein is captured onto Chitin agarose beads (New England Biolabs) via the CBD. After extensive washing of the beads with lyses buffer, the recombinant protein is recovered by thiol-induced on-column self-splicing of the intein tag by addition of Na-2-MESNA (2-mercaptoethane-sulfonic acid, sodium salt) to a final concentration of 50 mM and incubation for 12 hours at 4° (see e.g. New England Biolabs, 2002). Any co-eluted intein tag and uncleaved fusion protein are removed from the eluate in a second, subtractive affinity step. Finally, the protein is transferred into the appropriate storage buffer by elution through a gel filtration column (DG-10, Bio-Rad) previously equilibrated with the target buffer (HuR: 25 mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 800 mM NaCl, 0.2 % (w/v) Pluronic F-127; HuR12: 25 mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 136 mM NaCl), shock-frozen in small aliquots in liquid nitrogen and stored at –80 °.

c) Labeling:

The concentration of HuR_{12} is determined by UV-spectroscopy according to Gill S.C. et al., Anal.Biochem. 182, 319, 1989. The accurate concentration of HuR_{fl} is determined as follows: a sample containing ~200 µg HuR_{fl} is purified on a VYDAC C_{18} RP-HPLC column and eluted in a gradient of 100 % eluent A – 100 % eluent B in 40 minutes (A: 5 % CH_3CN, 95 % H_2O, 0.1 % (w/v) TFA; B: 80 % CH_3CN, 20 % H_2O, 0.1 % (w/v) TFA) with UV-detection at 280 nm and fluorescence detection (280 nm excitation/340 nm emission). The eluted fractions are pooled, lyophilised and dissolved at a concentration of ~ 20 µM in PBS (phosphate buffered saline; 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136 NaCl, 2.7 KCl, pH 7.2) containing 6M guanidinium . HCl. After determination of the concentration by UV-spectroscopy using the theoretical molar extinction coefficient for HuR at 280 nm in 6M guanidinium hydrochloride, this stock solution serves as external standard for a subsequent concentration determination of HuR samples by conventional RP-HPLC quantification. The quality of the purified protein is controlled by denaturing SDS-PAGE, UV-spectroscopy, analytical Size Exclusion Chromatography, RP-HPLC analysis, N-terminal sequencing, LC/ESI-MS (Liquid Chromatography/Electrospray Ionization-Mass Spectrometry) analysis and CD- (Circular Dichroism-) spectroscopy and western blotting with mouse monoclonal anti-HuR 19F12 (Molecular Probes), following standard protocols. According to LC/ESI-MS and RP-HPLC analysis, all HuR_{12} and HuR_{fl} preparations are >98% pure. For both proteins, N-terminal sequencing revealed the correct N-terminus but quantitatively missing Met_1. Results from Analytical Size Exclusion Chromatography further indicated soluble proteins without presence of higher aggregation states.

d) HuR – ARE binding assay by 2D-FIDA-anisotropy:

Binding of HuR or HuR1,2 to fluorescently labeled AREs and ARE-related RNA fragments is monitored by determination of the fluorescence anisotropy with 2dimensional-Fluorescence Intensity Distribution Analysis (2D-FIDA). Direct binding experiments are performed by titration of fluorescently labeled RNA fragments against increasing concentrations of recombinant HuR_{fl} or HuR_{12}. For this purpose, the fluorescently labeled RNA is first diluted to a concentration of ~ 5 nM in assay buffer (PBS, 0.1% Pluronic, 5 mM $MgCl_2$), thermally denatured by incubation for 2 min at 80°C and refolded by slow cooling to RT at -0.13 °C / sec in a thermocyler. Samples corresponding to the individual titration points (usually 11 points in a concentration range of 0.1 – 100 nM HuR_{fl} or 0.5 – 2500 nM HuR_{12} and a constant

RNA concentration of 0.5 nM) are prepared by mixing the appropriate volumes of assay buffer, RNA and protein solutions, at a final volume of 25 to 100 µl per sample.
The 2D-FIDA-r measurements are performed in 96 well glass bottom microtiter plates (Whatman) on an EvotecOAI PickoScreen 3 instrument at ambient temperature (constant at 23.5 °). The Olympus inverted microscope IX70 based instrument is equipped with two fluorescence detectors and a polarisation beam splitter in the fluorescence emission path, adopted to perform anisotropy measurements. A HeNe laser (λ = 543 nm, laser power = 495 µW) is used for fluorescence excitation. To ensure a high degree of polarisation of the fluorescence excitation source, an additional linear polarisation filter is placed in the optical path in front of the sample. The excitation laser light is blocked from the optical detection path by an interference barrier filter with OD (optical density) = 5. 5'-TMR (Molecular Probes) dissolved in assay buffer (c ~ 0.5 nM) is used for the adjustment of the confocal pinhole (70 µm) and for the determination of the G-factor of the instrument (see e.g. Lakowicz J.R, Principles of Fluorescence Spectroscopy, Plenum Publishers, New Yoprk, ed.2, 1999).
For the determination of the fluorescence anisotropy from the 2D-FIDA raw data, the Molecular Brightness q is extracted for each channel using the FIDA algorithm (see e.g. Kask P. et al., Biophys.J. 78, 1703, 2000 or Kask P. et al., PNAS 96, 13756, 1999). The anisotropy is then calculated as described in (Lakowicz J.R. cited above). The 2D-FIDA anisotropy signal is averaged for each sample from 10 consecutive measurements (à 5 seconds). After every 11 measured samples, measurements of 5'-TMR are performed for the determination of the G-Factor (calculated using $P_{(true)}$ TMR = 0.034).
For a determination of the equilibrium dissociation constant K_d, the recorded anisotropy data are fitted based on the exact algebraic solution of the binding equation describing the average steady-state anisotropy signal *r* in dependence of the degree of complex formation derived from the law of mass action:

$$r = r_{min} + \frac{(r_{max} * \frac{1}{Q} - r_{min}) * \left[(RNA_0 + HuR_0 + K_d) - \sqrt{(RNA_0 + HuR_0 + K_d)^2 - 4 * RNA_0 * HuR_0}\right]}{2 * RNA_0} \qquad [1]$$

wherein
$[RNA_{free}]$: equilibrium concentration of free RNA,
$[HuR_{free}]$: equilibrium concentration of free HuR,
[RNA-HuR]: equilibrium concentration of RNA-HuR12 complex,
$[RNA_0]$: total concentration of RNA,
$[HuR_0]$: total concentration of HuR,

r_{min}: .anisotropy of free RNA,

r_{max}: anisotropy of RNA-HuR complex,

r: average anisotropy for the steady-state equilibrium at the given HuR_0 and RNA_0 concentrations,

Q: quenching; for 2D-FIDA-anisotropy measurements, $Q = q_{tot(max)} / q_{tot(min)}$; at $q_{tot} = q_{II} + 2 * q_{\perp}$; q_{II}, $q_{\perp}$: molecular brightnesses in parallel and perpendicular polarisation channels.

The equation is compiled in the program GraFit 5.0.3 (Erithacus software, London) and the data are fitted based on least square regression using the Marquardt algorithm. All of the presented data are averages from at least three independent experiments.

For evaluations based on 2D-FIDA-r, optimal results are achieved at an average of ca. < 1 fluorescent particles in the confocal volume (see e.g. Evotec BioSystems AG, 2D-FIDA-Quick Guide, 2001), which corresponds to a fluorophore concentration of ca. 0.5 nM in the described setup. For this reason, the ligand (5'TMR-RNA) is diluted to a concentration of ~0.5 nM for all 2D-FIDA-r measurements. The accurate concentration in each sample is finally calculated based on a determination of the particle number in a parallel FCS (Fluorescence Correlation Spectroscopy) evaluation of the recorded 2D-FIDA-r data and the size of the confocal volume, as determined by the adjustment parameters for the point spread function.

e) RNA Secondary Structure Prediction:

RNA secondary structures of minimal free energy are predicted with RNAfold, suboptimal; structures with RNAsubopt which are part of the Vienna RNA Package (see e.g. Hofacker I.L. et al., Monatsh. Chemie 125, 167, 1994).

An RNA molecule of sequence s will adopt many secondary structures. The set of all secondary structures is called ensemble, $\varepsilon(s)$, and the frequency of each individual structure σ in the ensemble is determined by its stability, i.e. its energy $E(\sigma, s)$. The frequency of a particular structure σ_i can be calculated using Boltzmann's law:

$$p(\sigma_i, s) = \frac{e^{E(\sigma_i, s)/kT}}{\sum_{\sigma \in \varepsilon(s)} e^{E(\sigma, s)/kT}} = \frac{e^{E(\sigma_i, s)/kT}}{Q_s} \qquad [2]$$

Q_s is called the partition function of the secondary structure ensemble of sequence s. The probability of a particular structure element *A*, e.g. the probability to find NNUUNNUUU in single stranded conformation, in the ensemble is the probability of a set of structures. As the

probabilities of individual structures are roughly independent, the probability of the structure element *A* is simply the sum of the probabilities of all structures containing element *A*,

$$p(A,s)=\sum_{\sigma_i \in \varepsilon(A,s)} p(\sigma_i,s)=\frac{\sum_{\sigma_i \in \varepsilon(A,s)} e^{E(\sigma_i,s)/kT}}{Q_s} \qquad [3]$$

$$p(A,s)=\frac{Q_{A,s}}{Q_s} \qquad [4]$$

where $\varepsilon(A, s)$ denotes the ensemble of structures of sequence *s* constrained to structures containing *A* and $Q_{A,s}$ is the respective partition function.

Equation 3 is valid for structure elements which occur only once. Most elements of interest may occur repeatedly and possibly overlap. For a correlation with affinity data, only the discrimination *accessible* and *not-accessible* will be of interest. Consequently the definition of p is extended to the probability of structures containing at least one element A. A computation via the probability of its complement is not possible due to limitations for constrained folding. As the probabilities of substructures that may occur together are not independent, the sum of the individual probabilities has to be corrected for joint occurrences of A:

$$p(A_1 \cup A_2 \cup \ldots \cup A_{n-1} \cup A_n)=\sum_{i<n} p(A_i)-\sum_{i<j<n} p(A_i \cap A_j)+$$
$$\sum_{i<j<k<n} (A_i \cap A_j \cap A_k)-$$
$$\sum_{i<j<k<l} (A_i \cap A_j \cap A_k \cap A_l)+\ldots..$$

[5]

$$p(A_1 \cup A_2 \cup \ldots \cup A_{n-1} \cup A_n)=$$
$$\sum_{i<n} \frac{Q_{A_i,s}}{Q_s}-\sum_{i<j<n} \frac{Q_{A_i,A_j,s}}{Q_s}+\sum_{i<j<k<n} \frac{Q_{A_i,A_j,A_k,s}}{Q_s}-$$
$$\sum_{i<j<k<l<n} \frac{Q_{A_i,A_j,A_k,A_l,s}}{Q_s}+\ldots$$

[6]

Where $Q_{Ai,Aj,s}$ denotes the partition function of the ensemble constrained to structures containing elements A_i and A_j, $Q_{Ai,Aj,Ak,s}$ denotes the partition function of the ensemble constrained to structures containing elements A_i,A_j and A_k, etc.

To minimize numerical errors, the probabilities are calculated via the ensemble free energies W of ε(s) and ε(A, s).

$$W = -kT \ln Q \qquad [7]$$

$$p(A,s) = e^{\frac{W[\varepsilon(s)]-W[\varepsilon(A,s)]}{kT}} \qquad [8]$$

Ensemble free energies are computed using the Perl module of *RNAfold* which is part of the *Vienna RNA Package* (www.tbi.univie.ac.at/RNA/). The calculation of Q_s is straightforward; $Q_{A,s}$ is calculated by constraining the sequence positions corresponding to A to the particular structure.

Pseudocode for the calculation of p:

```
calc_p (sequence s, motif_position_list, motif_struct)
        wg = calc_enemle_free_energy (s)
        p = 0
        for I (1 .. number_fo_motifs)
                w_c = calc_ensemle_free_energy (s, motif I, motif_position_list, motif_struct)
                p+ = exp ((w_g – w_c)/kT)
                push (constrain_list, i)
                p+ = recurse (constrain_list, w_g)
        next
return p
recurse (constrain_list, w_g)
        pi = 0
        for j (last_element (constrain_list) .. number_of_motifs)
                push (constrain_list, j)
                w_c = calculate_ensemle_free_energy (s, constarin_list, motif_position_list,
                motif_struct)
                if (numer_elements (constrain_list)%2=0)
                        sign = +1
                else sign = -1
                p_i+ = sign * exp ((w_g – w_c)/kT)

                if (j <number_of_motifs)
                        p_i + = recurse (constrain_list, w_g)
                next
        return p_i
```

Example 2

Deduction of the HuR binding motif M:

Based on our previous observation that polyU is bound by HuR with high affinity, the effect of elongation of U_8 was tested. Individual RNA fragments are synthesized and the affinities (given as Kd_{app} values) to full length HuR are determined (see TABLE 1). While the simplest variant of U_8 motif (fragment No. 1) is not recognized by HuR, an elongation by one nucleotide to U_9 (fragment No.2) shows a sufficient high binding. An influence of the fluorescent dye is excluded by competition experiments with unlabeled RNA fragments. The 9mer fragment (fragment No.3) contains the HuD motif and an additional nucleotide 3`terminally but is not bound by HuR. The high affinity binding to fragment No.4 however indicates that non-U nucleotides are tolerated within HuR binding motif, but at certain positions only. We have found that 9 nucleotides are sufficient for binding of HuR and four different 9mer frames within $(AUUU)_3A$ are tested (see fragments No 4a) to 4d) in bold). The exclusive recognition of fragment 4b by HuR within the four corresponding fragments demonstrates that HuR binds to frame 2 within $(AUUU)_3A$. This frame is consistent with the HuD motif, but 5`terminally elongated by one uracil residue, suggesting the preliminary binding motif NN(U/C)UNN(U/C)U(U/C). Fragments 5, 6, 7a-7d and 8a-8c serve to tests tolerance for non-Uracil (exemplified by A=adenine) and C, respectively, at the depicted (bold and underlined) positions. In consequence we found that HuR sequence binding motif is NNUUNNUUU. This interaction appears to follow an all-or-nothing mechanism: While sequences with single mismatches are not recognized, sequences fulfilling this motif are bound with high affinity and an invariable Kd, Kd_{fund}, of 0.99 nM. Results are set out in TABLE 1 below.

TABLE 1

RNA fragment No	nucleotides	Kd_{app} (in nM)
1	U U U U U U U U U	not bound
2	U U U U U U U U U U	0.97 +/- 0.19
3	$(AUUU)_2A$	not bound
4a	$(AUUU)_3A$	1.40 +/- 0.39
4a	A U U U **A** U U U **A** U U U **A**	not bound
4b	A U U U **A** U U U **A** U U U A	0.77 +/- 0.25
4c	A U U U **A** U U U **A** U U U A	not bound
4d	A U U U **A** U U U **A** U U U A	not bound

RNA fragment No	nucleotides	Kd_{app} (in nM)
prel.consensus	N N U/C U N N U/C U U/C	
7a	U A A U U U U U U	not bound
7b	U A U A U U U U U	not bound
7c	U A U U U U A U U	not bound
7d	U A U U U U U A U	not bound
8a	U A C U U U U U U	not bound
8b	U A U U U U U U C	not bound
8c	U A U U U U C U U	not bound
5	U A U U A U U U U	1.14 +/- 0.24
6	A A U U U A U U U	1.01 +/- 0.27
MOTIF	N N U U N N U U U	

EXAMPLE 3a:

Designed mRNA secondary structure modulators mimic endogenous IL-2 response to CD28 stimulation in T cell activation.

To confirm the potential of designed modulators to influence the stability of mRNA and hence influence the regulation of gene expression *in vivo*, we select IL-2 mRNA regulation as a model. IL-2 is one example out of approximately 3000 genes ((Bakheet, et al 2001, Bakheet, Williams and Khabar 2003) which are specifically turned on and off by AU rich element (ARE) mediated degradation of the mRNA. As described above, ARE mediated mRNA regulation is dependent on a conformational switch in the ARE conformation. We demonstrate that the apparent affinity between the mRNA stabilizing protein HuR and an mRNA is directly proportional to the probability of structures presenting the HuR binding site NNUUNNUUU in single stranded conformation. In T-cell activation, the co-stimulatory signal (transduced via CD28) is known to explicitly trigger IL-2 expression by a stabilization of its mRNA (e.g. Powell, Ragheb et al., 1998, Shim, Lim et al., 2002). We investigate whether a controlled modulation of IL-2 mRNA secondary structure allows to mimic the endogenous downstream effect of CD28 stimulation in human primary T-cells.

a) IL-2 mRNA *opener* design

Positive modulators, henceforth denoted *openers*, are constructed for IL-2 to maximize the HuR binding site accessibility upon hybridization to the target mRNA sequence within the IL-2 3'UTR as sketched in Figure 1.

Two individual *openers* targeting the two HuR binding sites within the IL-2 3'UTR are selected for experimental verification (Figure 2, O_1 and O_2, Table 1).

TABLE 1:

Name		Sequence	Position
Openers	O_1	AATATAAAATTTAAATATTT	804-823
	O_2	TAGAGCCCCTAGGGCTTACA	909-928
Negative Controls	N_1	AGTGGGAAGCACTTAATTAC	757-775
	N_2	CATAATAATAAATATTTTGG	969-950

Positions refer to oligonucleotide hybridization sites within GenBank accession NM_000589.

The *opener* effect is initially validated *in vitro*. As measured in a 1D-FIDA mRNA-protein binding assay, HuR binds to the IL-2 3'UTR (281 nucleotides in length) with significantly higher affinity in presence of either of the *openers* (O_1: Kd_{app} = 11.80 (± 1.48) nM; O_2: Kd_{app} = 18.91 (± 1.91) nM; without *opener*: Kd_{app} = 32.77 (± 4.48) nM; Figure 2B). This increased HuR affinity correlates with the *opener* concentration reflecting the thermodynamics of the *opener* hybridization equilibrium (all specified Kds are optimum values). Negative controls are performed with two IL-2 3'UTR specific 20mers which do not alter the predicted accessibility *p(ssNNUUNNUUU)* (Figure 2A, N_1 and N_2). As confirmed experimentally, both oligonucleotides do not affect the HuR - IL-2 3'UTR interaction *in vitro* (N_1: Kd_{app} = 32.91 ± 6.34 nM; N_2: Kd_{app} = 32.77 ± 3.72 nM).

To verify that the *opener* oligonucleotides also function in an in-vivo situation, the increase of the endogenous HuR IL-2 mRNA association in a cellular system is measured. IL-2 mRNA - HuR complexes from lysates of CD3- versus CD3/CD28-stimulated human PBMC are co-immunoprecipitated in the absence and in the presence of the *opener*. HuR bound IL-2 mRNA is quantified by quantitative real-time RT-PCR. Indeed, both *openers* increase the level of HuR IL-2 mRNA association up to 9 fold in a concentration dependent manner (Figure 3).

The relevance of the IL-2 *openers* in ARE-dependent mRNA degradation is also examined. In human PBMC lysates IL-2 mRNA decay - which we found to be Mg^{2+} dependent - is monitored in the presence and in the absence of either *openers* O_1, O_2 as well as N_2. Upon Mg^{2+} addition the amount of remaining IL-2 mRNA is quantified over time by real-time RT-PCR. As demonstrated in Figure 4A and B, both *openers* allowed to block the rapid IL-2 mRNA degradation in a concentration dependent manner.

b) In vitro affinity measurements

The IL-2 3'UTR is prepared by run-off transcription from dsDNA templates with T7 RNA polymerase (T7 MEGASCRIPT in vitro transcription kit, Ambion). The T7 promoter is incorporated into the transcription templates during PCR amplification, using primers encompassing the 3'UTRs of IL-2 (nt 707-1035, GenBank accession numbers NM_000589). 1.5 to 2 nmol of the transcript are 3' terminally oxidized with Na(m-)IO_4 and coupled to a hydrazide activated fluorescent dye. The product is purified by RP-HPLC, desalted and transferred into aqueous solution by gel filtration (described e.g. in (Meisner, Uhl and Auer 2003)).

A 1:1 labeling stoichiometry is controlled by UV/VIS absorption spectroscopy.

Binding of HuR to the 3'terminally fluorescently labeled IL-2 3'UTR is measured in homogeneous solution and under true equilibrium conditions with 1D-FIDA (52, 53). Briefly, the labeled mRNA is titrated against increasing concentrations of recombinant HuR in presence and absence of *openers* or negative control oligodeoxynucleotides. HuR–mRNA complex formation is monitored by determination of the molecular brightness with 1D-FIDA The optical setup is analogous as already described earlier, but using one detection channel only and no polarization beam splitters in the optical paths. The molecular brightness q is extracted from the 1D-FIDA raw data using the FIDA algorithm and averaged from 20 consecutive measurements (10 s each). The molecular brightness data are fitted based on

Eq.3

$$q = q_{min} + \frac{(q_{max} - q_{min}) * \left|([RNA_0] + [HuR_0] + Kd_{app}) - \sqrt{([RNA_0] + [HuR_0] + Kd_{app})^2 - 4 * [RNA_0] * [HuR_0]}\right|}{2 * [RNA_0]}$$

[3]

with

$[RNA_0]$: total concentration of RNA,

$[HuR_0]$: total concentration of HuR,

Kd_{app}: apparent dissociation constant,

q_{min}: molecular brightness of free RNA,

q_{max}: molecular brightness of RNA-HuR complex,

q: average molecular brightness for the steady-state equilibrium at the given HuR_0 and RNA_0 concentrations; all presented data are averages from 20 individual FIDA measurements and representative for at least three independent experiments.

c) Preparation and stimulation of cells

Human PBMC are isolated from heparinized blood by Ficoll-Hypaque centrifugation, washed with PBS containing 0.15 % (w/v) bovine serum albumin (PBS/BSA), resuspended at 2×10^6 ml^{-1} in RPMI1640 (Gibco/BRL) supplemented with 10% (v/v) heat-inactivated FCS, 2mM L-glutamine, 100 µg ml^{-1} of streptomycin and 100 µg ml^{-1} of penicillin. PBMC are stimulated for 4 hours with PMA (25 ng ml^{-1}, Sigma-Aldrich) and anti-CD3 mAb (1µg ml^{-1}, Pharmingen) in absence and in the presence of anti-CD28 mAb (1µg ml^{-1}, Pharmingen) and incubated in a 37°C CO_2 incubator.

d) Co-immuneprecipitation of HuR-mRNA complexes

For each immuneprecipitation, 5×10^6 stimulated cells are washed with PBS/BSA and lysed at 4° in 100 µl hypotonic buffer (10 mM Tris/Cl pH 7.5, 10 mM NaCl, 10 mM EDTA, Protease Inhibitor (Complete Mini EDTA free Protease Inhibitor Cocktail, Roche; 3 tablets per 50 ml lysis buffer) and 0.5 % (v/v) Nonidet-P-40). RNAsin (0.4 u ml^{-1}, Pharmingen) Promega) and SuperasIn (0.2 u ml^{-1}, Ambion) are added to inhibit unspecific RNA degradation. The lysates are centrifuged at 4°C for 4 minutes at 15,000 x g to pellet nuclei. The cleared lysates are incubated for 5 minutes with 5 µg ml^{-1} of anti-HuR mAb (19F12, Molecular probes) at 4° in the presence and in the absence of 2.5 or 10 µM of *opener* O_1, O_2 or negative control oligonucleotides N_1, N_2. After addition of biotinylated anti(mouse) IgG mAb (10 µg ml^{-1}, Amersham Pharmacia), the immunecomplexes are captured on StreptavidinSepharose beads (Amersham Pharmacia). The beads are washed thoroughly with lysis buffer. HuR and the complexed mRNA are eluted with 50 mM Glycin/HCl pH 2.5, 50 mM NaCl (prewarmed to 95°). The eluates are passed by centrifugation through BioSpin gel filtration columns (BioRad), pre-equilibrated with H_2O. Co-precipitated RNA is quantified by real-time RT-PCR.

e) mRNA decay

5×10^6 stimulated PBMC are lysed in 250 µl of lysis buffer as described above, in the presence or in the absence of 10 µM of *opener* or negative control oligonucleotides O_1, O_2,

N_2. IL-2 mRNA degradation is initiated in the cleared lysates by addition of $MgCl_2$ to a net concentration of 5 mM free Mg^{2+}. The degradation reaction is proceedes at RT and is stopped after 7 minutes, 15 minutes, 30 minutes and 60 minutes incubation (50 µl aliquots for each timepoint) by addition of EDTA and guanidinium isothiocyanat containing buffer (Qiagen). RNA is isolated using the RNeasy Miniprep RNA isolation kit (Qiagen) according to the manufacturers protocol, with DNAse I treatment for elimination of residual DNA.

f) Quantitative real-time RT PCR

RNA is reverse transcribed to cDNA using the TaqMan RT PCR reagents (Applied Biosystems) and random hexamers for priming following standard protocols. Quantitative RT-PCR is performed with SYBR Green detection on an ABI7700 instrument (Applied Biosystems) with IL-2 specific primers (forward: 5'-TCACCAGGATGCTCACATTTAAGTT-3; reverse: 5'-GGAGTTTGAGTTCTTCTTCTAGAC ACTGA-3'; primers are a gift from F. Kalthoff, Novartis Institute for Biomedical Research Vienna). EF-1 alpha is used as endogenous control for normalization
(primers: forward 5'-TTTGAGACCAGCAAGTACTATGTGACT-3', reverse 5'-TCAGCCTGAGATGTCCCTGTAA -3'). The ΔΔCt method is used for relative quantification of IL-2 mRNA levels (as described by Applied Biosystems). All presented data are averages from at least 5 identical independent samples and representative of at least two independent experiments using cells from different donors.

Example 3b)

Analogously as described in example 3a), but using TNF-α mRNA instead of IL-2 mRNA and the appropriate reagents the following oligonucleotides are identified:

SEQ ID No 9: TCGGCCAGCTCCACGTCCCG,
SEQ ID No 10: TCTGGTAGGAGACGGCGATG;
SEQ ID No 11: ACGGCGATGCGGCTGATGGT;
SEQ ID No 12: TTCTGGAGGCCCCAGTTTGA,
SEQ ID No 13: ATTCCAGATGTCAGGGATCA, and
SEQ ID No 14: ATCACAAGTGCAAACATAAA.

In measuring mRNA decay as described in example 3e) it could be shown that oligonucleotides of SEQ ID No 9 to 14 enhance TNF-α mRNA stability and thereby confirm their function as openers.

Patent claims

1. Method for modulating regulatory RNA-ligand interactions comprising
   (a) defining and selecting a secondary structure element of an RNA molecule which is required for the recognition by a ligand, e.g. protein,
   (b) calculating the thermodynamic probability of the secondary structure element of step a) in the secondary structure ensemble of said RNA,
   (c) calculating the thermodynamic probability of the secondary structure element of step a) in the secondary structure ensemble of said RNA hybridized to a reverse complementary oligonucleotide,
   (d) determining an oligonucleotide that changes the thermodynamic probability of said secondary structure element beyond a defined probability threshold,
   (e) providing an oligonucleotide as determined in step (d),
   (f) hybridizing an RNA comprising said secondary structure element of step (a) to an oligonucleotide of step (e), and
   (g) determining the effect of said hybridization on the thermodynamic probability of said secondary structure element.

2. Method of claim 1, wherein said secondary structure element is defined and selected by a method comprising
   (a) providing an RNA-binding ligand LIG,
   (b) providing at least 3 different RNAs of sequences s_{M1} s_{M2}, and s_{M3}, each of s_{M1} s_{M2}, and
   s_{M3} having the same sequence binding motif MOT for the ligand LIG, but each differing in the secondary structure,
   (c) calculating each $p(s_M,\sigma_i)$ for each s_{M1} s_{M2}, and s_{M3} which is the probability that a structure contains at least one MOT in a particular test conformation σ_i, e.g. calculating each $p(S_M,\sigma_i)$ according to the equation [2], as set out below,
   (d) measuring each macroscopic affinity constant Kd_{app} for the binding of LIG to each of s_{M1} s_{M2}, and s_{M3},
   (e) determining for any test conformation σ_i whether the correlation between each of Kd_{app} and $p(s_M, \sigma_i)$ for each of s_{M1} s_{M2} and s_{M3} follows the dependency
   $Kd_{app} \sim 1 / p(s_M, \sigma_i)$,
   and

(f) choosing a secondary structure element of conformation σ_a which follows said dependency.

3. A method of claim 1 or 2, further comprising the steps of

(g) determining the fundamental affinity constant Kd_{fund} of LIG to MOT in an active conformation σ_a based on the equation $Kd_{app} = Kd_{fund} / p(s_M, \sigma_a)$, and

(h) (optionally) providing, e.g. novel, sequences with a defined Kd_{app} for the ligand LIG.

4. Method of any one of claims 1 to 3, wherein the RNA is a TNF-α mRNA, the ligand is ELAVL1 and the oligonucleotide has a sequence selected from the group consisting of

SEQ ID No 9: TCGGCCAGCTCCACGTCCCG,
SEQ ID No 10: TCTGGTAGGAGACGGCGATG;
SEQ ID No 11: ACGGCGATGCGGCTGATGGT;
SEQ ID No 12: TTCTGGAGGCCCCAGTTTGA,
SEQ ID No 13: ATTCCAGATGTCAGGGATCA, and
SEQ ID No 14: ATCACAAGTGCAAACATAAA.

5. Use of a method of any one of claims 1 to 4 for manipulating the expression of a gene by altering the secondary structure of the corresponding RNA.

6. Assay for identifying an agent that modulates the effect of the hybridization of an RNA molecule to an oligonucleotide comprising

(a) hybridizing an RNA comprising a secondary structure element which is required for recognition by a ligand to an oligonucleotide that changes the thermodynamic probability of said secondary structure element beyond a defined probability threshold in the presence and in the absence of a candidate compound,

(b) determining the effect of hybridization of said RNA to said oligonucleotide in the presence and in the absence of said candidate compound,

(c) identifying an agent which modulates the effect of hybridization.

7. Assay for identifying an agent that mimics the effect of hybridization of an RNA molecule to an oligonucleotide comprising

(a) hybridizing an RNA comprising a secondary structure element which is required for recognition by a ligand to an oligonucleotide that changes the thermodynamic

probability of said secondary structure element beyond a defined probability threshold

(b) hybridizing an RNA comprising a secondary structure element which is required for recognition by a ligand to a candidate compound which is expected to have a similar effect as the oligonucleotide,

(c) determining the effect of hybridization for steps (a) and (b), and

(d) identifying an agent which mimics the effect of hybridization of step (a).

8. Assay of any one of claims 6 or 7, wherein the effect of hybridization is determined by measuring a signal which is related to the effect of hybridization, which effect is selected from the group consisting of changes in secondary RNA structure, tertiary RNA structure, RNA-ligand affinity, RNA oligo- or multimerization, ligand oligo- or multimerization, conformational change of the ligand, efficiency of a downstream effect of RNA-ligand recognition, RNA splicing, covalent RNA modifications, RNA localization, RNA stability, RNA translation and protein expression profiles.

9. Assay of claim 6 to 8, wherein the RNA is an mRNA.

10. Assay of any one of claims 6 to 9, wherein the RNA, the ligand and the oligonucleotide are as defined in claim 4.

11. Use of an assay of any of claims 6 to 10 for high throughput screening.

12. Agent identified by an assay of any one of claims 5 to 10 for use as a pharmaceutical.

13. An oligonucleotide that changes the thermodynamic probability of a secondary structure element beyond a defined probability threshold identified by a method of any one of claims 1 to 4.

14. An oligonucleotide of claim 13 having a sequence selected from the group consisting of

SEQ ID No 9: TCGGCCAGCTCCACGTCCCG,

SEQ ID No 10: TCTGGTAGGAGACGGCGATG;

SEQ ID No 11: ACGGCGATGCGGCTGATGGT;

SEQ ID No 12: TTCTGGAGGCCCCAGTTTGA,

SEQ ID No 13: ATTCCAGATGTCAGGGATCA, and

SEQ ID No 14: ATCACAAGTGCAAACATAAA.

15. Pharmaceutical composition comprising an agent identified by a method of any of claims 1 to 4 or an oligonucleotide of any one of claims 13 or 14 beside at least one pharmaceutical excipient.

16. Use of an oligonucleotide of any one of claims 13 or 14 for manipulating regulatory RNA-ligand interactions.

17. Use of an oligonucleotide of any one of claims 13 or 14 for influencing the stability of an RNA molecule.

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Abstract

The present invention provides a method for modulating regulatory RNA-ligand interactions, assays for identifying agents and an oligonucleotide that changes the thermodynamic probability of a secondary structure element of an RNA molecule.